(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 June 2002 (13.06.2002)

PCT

(10) International Publication Number
WO 02/46476 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/US01/50997

(22) International Filing Date: 26 October 2001 (26.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/698,387 27 October 2000 (27.10.2000) US

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(81) Designated States *(national)*: CA, JP.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/46476 A2

(54) Title: GENOTYPING USING PARTIALLY-COMPLEMENTARY PROBES

(57) Abstract: Methods for detecting at least two distinct target sequences in a test sample are provided. The methods employ probes that are not completely complementary to one another, but nevertheless hybridize to each other, to detect the distinct target sequences. The target sequences can be amplified prior to detection with the probes. Variant forms of a single sequence are particularly suited for detection according the methods provided.

GENOTYPING USING PARTIALLY-COMPLEMENTARY PROBES

Technical Field

The present invention relates to detecting nucleic acid sequences and, in particular, relates to detecting distinct nucleic acid sequences with probes that are complementary to such distinct nucleic acid sequences, as well as complementary to each other.

Background of the Invention

Studies designed to determine the genomic sequence of various organisms, as well as studies designed to compare genomic sequences, have elicited information regarding polymorphisms in various genomes ranging from the human genome to the HIV genome. A wide variety of polymorphisms in the genomes of such organisms have previously been described. The various types of genetic polymorphsims include single base substitutions; insertions or deletions; variable numbers of tandem repeats; deletions of all or a large part of a gene; gene amplifications; and chromosomal rearrangements. Generally, polymorphisms that involve a single nucleotide are called single nucleotide polymorphisms or "SNPs". In the event a genome contains a particular SNP, a sequence from the region containing the SNP may exist in one of two or more forms. If one of the forms can be identified as occurring in the majority of the population, or if it can be associated with full functionality of a protein product, it is referred to as the "wild-type" sequence. The other form(s) of the SNP would be referred to as "mutant".

Some variations in certain genes are responsible for various disease states. For example, venous thrombosis is a fairly common disorder that annually affects approximately 1 in 1000 people. In some cases, thrombosis can be traced to SNPs in genes encoding proteins that participate in the cascade of events responsible for blood clotting. For example, the so-called "factor V Leiden" mutation, a mutation in the gene encoding factor V, has been reported to be a factor responsible for thrombosis. Additionally, a variation in the 3' untranslated region of the prothrombin gene also has been reported to be responsible for thrombosis. Persons having either, or both, of the above gene variations have been reported to be at an even greater risk of thrombosis in the event that they are, for example, undergoing surgery, pregnant, or taking oral contraceptives.

In certain cases, a mutant sequence and wild type sequence can be contained in a single genome. For example, such a case can arise where a sequence is derived from an organism that contains more than one set of chromosomes (variously referred to as "polyploidy"). Additionally, organisms containing a single chromosome may also contain variant forms of more than one gene in cases where the chromosome contains a gene amplification. While a genome may contain duplicate copies of either the wild-type or mutant version of the sequence in question (variously referred to as "homozygous" for the particular sequence), a further possibility is that the genome contains a copy of each of the wild-type and mutant sequence (variously referred to as "heterozygous" for the particular sequence). The existence of closely related sequences presents several challenges when attempting to detect these sequences in a test sample.

Previously, detecting related sequences in a test sample was performed using RFLP and gel electrophoresis. Dual and separate amplification reactions to determine the presence of a mutant and wild type sequence in a sample have also been performed to detect the presence of multiple related sequences in a test sample. For example, genetic tests for the factor V Leiden and the prothrombin mutations have been reported in the literature. Many of the previously reported genetic based assays are performed with the aid of an amplification reaction such as the polymerase chain reaction (PCR) or the ligase chain reaction (LCR). After amplification of the regions suspected of containing the particular mutation, the amplification products are often times detected using gel electrophoresis. Amplification based assays for the above mutations are run individually and separately with one reaction designed to detect the wild type sequence and the other reaction designed to detect the mutant sequence. Accordingly, these assays can be cumbersome and expensive to perform. Hence, it would be useful to provide assays and assay reagents that allow an assay for two related sequences to be run in a single reaction vessel. Such reagents and methods would therefore avoid the use of dual and separate reactions and the use gel electrophoresis for purposes of detecting related sequences in a test sample.

Brief Description of the Invention

The present invention provides a method for detecting at least one of two related target nucleic acid sequences in a test sample. The method can be employed with nucleic acid amplification techniques that advantageously can be performed in a single reaction

vessel. Generally, the method comprises the steps of contacting a test sample with a first and second probe. The probes are designed such that (i) they are completely complementary to the each other except for at least one mismatch, (ii) the first probe is more complementary to a sense strand of the target sequence than to the second probe, (iii) the second probe is more complementary to an anti-sense strand of a second target sequence than to the first probe, and (iv) the probes hybridize to each other at an ambient temperature. A target sequence/probe hybrid is formed by hybridizing at least the first or second probe to the first or second target sequence. The so-formed hybrid is detected as an indication of the presence of one of the related target sequences in the test sample. Reagents for performing the above method are also provided.

Detailed Description of the Invention

The present invention provides methods for detecting two distinct target sequences in a test sample using a pair of probes that hybridize to one another but are not completely complementary to each other. Generally, the method comprises, if necessary, amplifying target sequences using any of the well known amplification techniques and detecting amplified target sequences, if any, with the probes. Advantageously, the distinct target sequences often times can be amplified using common amplification reagents. Under certain circumstances, such as when the target sequence is present in sufficient concentration, the target sequence may not require amplification before being detected with the probes. The probes generally are designed such that they are completely complementary to their respective target sequences and are sufficiently complementary to each other to hybridize. Typically, the probes have at least one mis-matched base pair preferably located between the 5' and 3' terminal nucleotides of the respective probes. The use of probes that are more complementary to their respective target sequences than to each other has been found to enable the detection of distinct, but related, target sequences in a single test sample without the need to introduce separate probes to the products of separate amplification reactions. The invention is particularly suited for detecting the presence of variant forms of a nucleic acid sequence in a test sample.

A "target sequence" as used herein means a nucleic acid sequence that is detected, amplified, both amplified and detected, or otherwise is complementary to the amplification primers or one of the probes. Additionally, while the term target sequence is often used

herein to refer to a single strand of nucleic acid, those skilled in the art will recognize that the target sequence can also be double stranded. As previously mentioned, target sequences that can be detected using the method provided herein are related. Such target sequences are related insofar as one target sequence is usually a variation of the other target sequence. In particular, the target sequences that can be detected typically have the same sequence except that one of the target sequences contains, for example, a polymorphism that makes its sequence composition distinct from the other target sequence. Thus, for example, a first target sequence and second target sequence may be the same except that the second target sequence contains one or more nucleotide substitutions, deletions, and/or insertions. Target sequences may be derived from any organism containing a nucleic acid genome.

The term "test sample" as used herein, means anything suspected of containing a target sequence. The test sample can be derived from any biological source, such as for example, blood, bronchial alveolar lavage, saliva, throat swabs, ocular lens fluid, cerebral spinal fluid, sweat, sputa, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, amniotic fluid, tissues such as heart tissue and the like, or fermentation broths, cell cultures, chemical reaction mixtures and the like. The test sample can be used (i) directly as obtained from the source or (ii) following a pre-treatment to modify the character of the sample. Thus, the test sample can be pre-treated prior to use by, for example, preparing plasma from blood, disrupting cells, preparing liquids from solid materials, diluting viscous fluids, filtering liquids, distilling liquids, concentrating liquids, inactivating interfering components, adding reagents, purifying nucleic acids, and the like.

A "primer sequence", "primer sequences" or "primer(s)" refers to reagents, most typically short strands of nucleic acid or "oligonucleotides", that prime synthesis of multiple copies of a target sequence. Oligonucleotides vary greatly in size but typically are in the range of between 10 and 1000 nucleotides in length, more typically in the range of between 15 and 100 nucleotides in length. Amplification reactions that employ primer sequences to synthesize multiple copies of a target sequence (or amplify the target sequence) are, by now, well know in the art. LCR described in European Patent Number 320 308 and its variations, such as gap LCR described in U.S. Patent Number 5,792,607 (herein incorporated by reference), NASBA or similar reactions such as TMA described in U.S. Patent Number 5,399, 491 (herein incorporated by reference), and preferably PCR, and variations of PCR, which are described in U.S. Patents Numbered 4,683,195, 4,683,202, and 5,310,652 (all of

which are herein incorporated by reference) are examples of amplification reactions that can be employed according to the present invention.

The phrase "amplification reaction reagents" as used herein means reagents which are well known for their use in nucleic acid amplification reactions and may include but are not limited to: a single or multiple reagent, reagents, enzyme or enzymes separately or individually having reverse transcriptase, polymerase, and/or ligase activity; enzyme cofactors such as magnesium or manganese; salts; nicotinamide adenine dinucleotide (NAD); and deoxynucleoside triphosphates (dNTPs) such as, for example, deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and thymidine triphosphate. The exact amplification reagents employed are largely a matter of choice for one skilled in the art based upon the particular amplification reaction employed.

Probe sequences are similar to primer sequences insofar as they hybridize to target sequences, facilitate detection of target sequences, and typically are oligonucleotides. Probes generally are nucleic acid or they can be composed of nucleic acid analogs such as, for example, uncharged alkyl analogs such as methyl phosphonates, and phosphotriesters, peptide nucleic acids (as is disclosed in WO 93/25706), and morpholino analogs (as disclosed in U.S. Patents 5,142,047, 5,235,033, 5,166,315, 5,217,866 and 5,185,444). Additionally, the term probe is intended to mean that portion, or that group of consecutive nucleotides, of a nucleic acid sequence that is predetermined or designed to hybridize to its target sequence, as those skilled in the art will recognize that additional nucleotides can be added to a probe for other purposes. For example, such probe extensions, or tails, are commonly added for purposes of assisting in detecting or purifying the probe.

Probes are provided in pairs and each probe of a particular pair hybridizes to the other member of the pair. The probes, however, are not perfectly complementary to each other which means that there is at least one mis-matched base pair on each probe. A "mis-matched base pair" (variously referred to as simply a "mismatch") means that one or more nucleotides on one probe does not hydrogen bond in the typical Watson-Crick fashion with one or more corresponding nucleotides on the other probe. Generally, probes are more complementary to their respective target sequences than they are to one another. Preferably, however, the probes are designed in such a manner so as to be perfectly complementary to distinct target sequences and therefore each nucleotide on a particular probe finds a corresponding

complementary nucleotide on its target sequence making the probe completely complementary with its target.

There are numerous configurations that the probes may take and the exact probe configuration is largely dependent upon particular target sequences selected. Specifically, mis-matches between the probes that make them partially non-complementary can occur anywhere and to any extent along the length of the probes. The probes, however, should be configured such that they hybridize to each other at ambient temperatures (e.g. 15°C - 30°C) in the absence of their respective target sequences but preferentially bind to the respective target sequences when they are present. Probes can be designed such that they hybridize to each other at ambient temperatures but preferentially bind to a target sequence when it is present (at any temperature where hybridization can occur) using well known principles of hybridization or annealing kinetics. Hybridization is dependent in a rather predictable manner on several parameters, including temperature, ionic strength, sequence length, complementarity, and G:C content of the sequences. For example, lowering the temperature in the environment of complementary nucleic acid sequences promotes annealing. For any given set of sequences, melt temperature, or "Tm", can be estimated by any of several known methods. Ionic strength or "salt" concentration also impacts the melt temperature, since small cations tend to stabilize the formation of duplexes by negating the negative charge on the phosphodiester backbone. Typical salt concentrations depend on the nature and valency of the cation but are readily understood by those skilled in the art. Similarly, high G:C content and increased sequence length are also known to stabilize duplex formation because G:C pairings involve 3 hydrogen bonds where A:T pairs have just two, and because longer sequences have more hydrogen bonds holding the sequences together. Thus, a high G:C content and longer sequence lengths impact the hybridization conditions by elevating the Tm.

Once probes are selected for a given set of target sequences, the G:C content and length will be known and can be accounted for in determining precisely what the hybridization conditions will encompass. Since ionic strength is typically optimized for enzymatic activity, the next most obvious parameter to vary is the temperature. Generally, the Tm of hybridized probes will be less than the Tm of the hybrid product formed between the probes and their respective target sequences. Thus, obtaining a suitable probe configuration is well within ordinary skill of one practicing this art. Additionally, determining whether a given probe configuration meets the above criteria can be

accomplished empirically. For example, the hybridization characteristics for a particular set of probes, and their respective target sequences, can be determined by raising and lowering the temperature in the environment of the probes and the probes and target. Generally speaking, given the fact that the probes are selected such that they are more complementary to their respective targets, complementary probes inherently will have a lower Tm than the Tm between the probes and their respective target sequences.

The exact size of the probes is largely dependent upon the target sequences and the number of nucleotides involved in the variation between the two sequences. Preferably, however, the probes are between 10 and 1000 nucleotides long, more preferably between 10 and 100 nucleotides long, and most preferably between 10 and 50 nucleotides long. The probes hybridize to one another except in regions containing the variance between the two target sequences for which the probes are respectively specific. The region or regions of the probe that are not complementary also can be of any length as long as the probes also have regions that are sufficiently complementary to permit the probes to hybridize at ambient temperatures. Regions of the probes that are not complementary also can be located at any position in the probes as long as the probes hybridize to one another at ambient temperatures. Typically, the probes are employed to hybridize to target sequences containing "small variations" or "small polymorphisms" such as, for example, base substitutions, deletions, or insertions involving ten or less nucleotides, more typically less than five nucleotides, preferably less than three nucleotides, and most preferably a single nucleotide polymorphism.

Also, while the region or regions of the probes that are not complementary can be located at any region of the probes, it is preferable to have these regions internal to the 5' and 3' terminal nucleotides of the probes, except in cases where one or both probes contain an overhang which is discussed in detail below. The terminal nucleotides are those that are the last nucleotides on either end of the probe that are designed to hybridize to the target sequence. More preferably, the region or regions of the probes that are non-complementary are located between the terminal nucleotide and the center nucleotide or nucleotides of the probes. As used herein, the term "center nucleotide(s)" is intended to mean the nucleotide or nucleotides that are located in the middle of a particular probe. For example, in the case of a five nucleotide probe, the center nucleotide would be the third nucleotide in from the 5' terminal nucleotide. In the case of a six nucleotide probe, the center nucleotides would be the third and fourth nucleotides in from the 5' terminal nucleotide. Hence, it is preferable to

locate the region or regions of the probes that are not complementary off center (e.g. closer to the 5' end of one probe). Locating a mismatch off center is particularly preferred in cases where the mismatch is a result of the variation between the target sequences and is the only mismatch between the probes.

Given the above, when the probes are hybridized to one another, several probe pair configurations are possible. Preferably, the probes are not co-extensive insofar as the 3'ends of the probes do not completely align with the 5' ends of the complementary probe when they are hybridized to one another. In other words one probe may be longer than the other at one end, which is commonly referred to in the art as an "overhang." Hence, an overhang at the 3'end of one probe means that it extends by one or more nucleotides past the 5' end of its complementary probe. Preferably, one of the probes contains an overhang on the 3' end. Alternatively, it is preferred to have a 3' overhang on both probes.

While probes generally are designed such that they are completely complementary to their respective target sequences, probes may have mis-matches with their respective target sequences as long as the number of mismatches between the probes is sufficient to meet the above criteria (i.e. the probes hybridize to each other at ambient temperatures but preferentially bind their respective targets). Probes also may have mis-matches with their respective target sequences when they are functionalized with, for example, nucleic acid tails that are not complementary to the target sequence. As previously mentioned, tails are typically used, for example, for purposes of detecting hybrids formed between probes and their respective target sequences.

As previously mentioned, the probes hybridize to distinct but related target sequences. Specifically, a first probe hybridizes to a first target sequence and a second probe hybridizes to a variant form of the first target sequence that is complementary to the first target but for the existence of a variation between the first target sequence and second target sequence. Accordingly, probes are designed such that they hybridize to opposite strands of related target sequences. Thus, for example, in the event the target sequences were double stranded, a first probe would be complementary to the sense strand of the first target sequence and the second probe would be complementary to the anti-sense strand of the second target sequence. Additionally, notwithstanding the fact that the target sequence, or its variant form, may not be present in a test sample, the probes nevertheless are pre-designed to hybridize to both forms of target sequence. Variant forms of a target sequence may include, for example, a first

target sequence and the same sequence that contains one or more nucleotide substitutions, deletions, and/or insertions. Hence, for example, a pair of probes may contain a single nucleotide mismatch when hybridized to one another when the target sequences are the same but for a single nucleotide polymorphism such as a single base pair substitution.

Various target sequences and variations of such target sequences can be detected using the probes provided herein. For example, the respective target sequences to which the probes are complementary could be from a single stranded RNA viral genome and a variant or mutated strain of that particular viral genome. Specifically, the first probe could be complementary to a region of the single stranded genome as it normally occurs and the second probe could be complementary to the opposite strand of the same genome that contains a mutation such as a single base pair substitution. In this particular case, it will be understood that the RNA may first be reverse transcribed into DNA and the strand to which the second probe hybridizes will be present as a result of amplification of the cDNA strand reverse transcribed from the RNA genome. Methods for reverse transcribing and amplification have been described above. Additionally, according to this embodiment, it is possible that only one version of the virus is present in the test sample. Accordingly, only one probe will hybridize to its target.

Alternatively, the original target sequences may be derived from the human genome. In this case, a first probe could be complementary to the sense strand of a gene (the "wild-type sequence") and the second probe could be complementary to the antisense strand of the same gene containing two single base substitutions (the "mutant sequence"). Depending upon the genotype of the individual sample, only the first probe would hybridize to its target if the genotype was homozygous for the wild-type sequence, both the first and second probes would hybridize to their targets if the genome was heterozygous, and only the second probe would hybridize if the genome were homozygous mutant.

Primer and/or probe sequences can be labeled for purposes of detecting hybrids formed between the target sequences and probes using any of the well known labels or labeling chemistries and is largely a matter of choice for those skilled in the art. Techniques for labeling sequences are well known and include, for example, techniques described in U.S. Patent Numbers 4,762,779; and 4,948,882, both of which are herein incorporated by reference. Typically, primer and probe sequences are labeled such that a hybrid or hybrids formed between the target sequences and complementary probes are distinguishable. In other

words, complementary probes are labeled so that a first probe/target sequence hybrid can be distinguished from a second probe/target sequence hybrid. Various configurations for making the above distinction are well known and also a matter of choice for those skilled in the art based upon the choice of detection apparatus.

The term "label" as used herein means a molecule or moiety having a property or characteristic which is capable of detection. A label can be directly detectable, as with, for example, radioisotopes, fluorophores, chemiluminophores, enzymes, colloidal particles, fluorescent microparticles and the like; or a label may be indirectly detectable, as with, for example, specific binding members. It will be understood that directly detectable labels may require additional components such as, for example, substrates, triggering reagents, light, and the like to enable detection of the label. When indirectly detectable labels are used, they are typically used in combination with a "conjugate". A conjugate is typically a specific binding member which has been attached or coupled to a directly detectable label. Coupling chemistries for synthesizing a conjugate are well known in the art and can include, for example, any chemical means and/or physical means that does not destroy the specific binding property of the specific binding member or the detectable property of the label. As used herein, "specific binding member" means a member of a binding pair, i.e., two different molecules where one of the molecules through, for example, chemical or physical means specifically binds to the other molecule. In addition to antigen and antibody specific binding pairs, other specific binding pairs include, but are not intended to be limited to, avidin and biotin; haptens and antibodies specific for haptens; complementary nucleic acid or nucleic acid analog sequences; and the like.

Detection platforms that can be employed to detect the hybrids formed between the complementary probes and their respective target sequence include any of the well known homogeneous or heterogeneous techniques well known in the art. Examples of homogeneous detection platforms include the use of FRET labels attached to probes that emit a signal in the presence of the target sequence. So-called TaqMan assays described in U.S. Patent Number 5,210,015 (herein incorporated by reference) are examples of techniques that can be employed to homogeneously detect nucleic acid sequences. Briefly, for example, according to a TaqMan format one probe could be labeled with a first fluorophore that emits a signal at a first wavelength and an appropriate quenching entity that is capable of quenching the signal from the first fluorophore. The second probe could be labeled with a second fluorophore that

emits a signal at a second wavelength and an appropriate quenching entity that is capable of quenching the signal from the second fluorophore. According to the principles of TaqMan-type assays the probes would be cleaved in the presence of their respective targets during the course of an amplification reaction using an enzyme having 5' to 3' exonucleolytic activity. As a result, as many as two distinct signals could be detected or as few as no signals could be detected depending upon the presence of the target(s).

Heterogeneous formats typically employ a capture reagent to separate amplified sequences from other materials employed to amplify and/or detect a target sequence. Capture reagents typically are a solid support material that is coated with one or more specific binding members specific for the same or different binding members. A "solid support material", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. Solid support materials thus can be a latex, plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface or surfaces of test tubes, microtiter wells, sheets, beads, microparticles, chips, and other configurations known to those of ordinary skill in the art. An exemplary capture reagent includes an array which generally comprises oligonucleotides or polynucleotides immobilized to a solid support material in a spatially defined manner.

For example, a heterogeneous format that could be employed to detect probes hybridized to their respective target sequences could include first and second probes that are labeled with first and second binding members. The primers employed to amplify the respective target sequences could be labeled with a third binding member. Once the probes were hybridized to the target sequences, if present, to form target sequence/probe hybrids, the hybrids could be immobilized to a solid support using the third binding member attached to the primer sequences. If desired, the solid support could be washed to remove excess reagents, and the hybrids, if any, could be detected using conjugates having distinct directly detectable labels. Signals, if any, associated with the solid phase could then be detected to indicate the presence of target sequences in the test sample.

According to a preferred embodiment, "oligonucleotide hybridization PCR" (variably referred to herein as "OH PCR") as described in U.S. Patent Application Serial No. 08/514,704, filed August 14, 1995, that is herein incorporated by reference, is employed. Briefly, the reagents employed in the preferred method comprise primers, complementary probes, as well as amplification reagents for performing an amplification reaction. The

primers are employed to prime extension of a copy of a target sequence (or its complement), and are labeled with either a capture label or a detection label. The probe sequences are used to hybridize with the sequences generated by the primers, and typically hybridize with a sequence that does not include the primers. Similarly to the primers, the probes are also labeled with either a capture label or a detection label with the caveat that when the primer is labeled with a capture label the probe is labeled with a detection label and vice versa. Detection labels have the same definition as "labels" previously defined and "capture labels" are typically used to separate extension products, and probes associated with any such products, from other amplification reactants. Specific binding members (as previously defined) are well suited for this purpose. Also, probes used according to this method are preferably blocked at their 3' ends so that they are not extended under hybridization conditions. Methods for preventing extension of a probe are well known and are a matter of choice for one skilled in the art. Typically, adding a phosphate group to the 3' end of the probe will suffice for purposes of blocking extension of the probe.

According to the above preferred embodiment, the probes initially are part of the reaction mixture. Additionally, it is preferred to select primers, probes and amplification conditions such that the probe sequence has a lower melt temperature than the primer sequences so that upon placing the reaction mixture under amplification conditions copies of the target sequence or its complement are produced at temperature above the Tm of the probe. After such copies are synthesized, they are denatured and the mixture is cooled to enable the formation of hybrids between the probes and any copies of the target or its complement. The rate of temperature reduction from the denaturation temperature down to a temperature at which the probes will bind to single stranded copies is preferably quite rapid (for example 8 to 15 minutes) and particularly through the temperature range in which an enzyme having polymerase activity is active for primer extension. Such a rapid cooling favors copy sequence/probe hybridization rather that primer/copy sequence hybridization and extension.

As indicated above, various target sequences and variant forms of the target sequence can be detected in a single reaction vessel. For example, the portion of the gene encoding factor V where the factor V Leiden mutation occurs and the factor V Leiden mutation could be employed as the target sequence and the variant form of the target sequence respectively. Alternatively, the 3' untranslated region of the prothrombin gene susceptible to containing

the mutation causitive of thrombosis and the same region that contains the mutation could be employed as the target sequence and the variant form of the target sequence. Based upon the sequence of these genes and the known mutations, probes completely complementary to, for example, the sense strand of the wild type sequence and anti-sense strand of the variant or mutant sequence could be synthesized with or without overhangs as mentioned above. Given the nature of the above mutatations (i.e. single base substitutions) such probes at least would have a mismatch where the mutation occurs and nevertheless be sufficiently complementary to each other to hybridize at ambient temperatures while preferentially hybridizing to their respective target sequences. Exemplary, probe pairs meeting the above criteria for the factor V gene and factor V Leiden mutation include SEQ. ID. NO. 5 and any one of SEQ. ID. NO. 6, SEQ. ID. NO. 7, SEQ. ID. NO. 8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, or SEQ. ID. NO. 11. Exemplary probe pairs meeting the above criteria for the 3' untranslated region of the prothrombin gene and mutation to the 3' untranslated region of the prothrombin gene include SEQ. ID. NO. 16 and any one of SEQ. ID. NO. 17, SEQ. ID. NO. 18, or SEQ. ID. NO. 19.

Probe pairs according to the invention, such as those exemplified above or otherwise meet the criteria stated above can be employed in assays to detect a target sequence and its variant (e.g. the factor V gene, or a part thereof which may contain the factor V Leiden mutation, and the portion that actually contains the factor V Leiden mutation). In particular, a test sample suspected of containing the target sequence and a variant form of the target sequence can be contacted with the probe pair in a single reaction vessel. Under suitable conditions known to those skilled in the art, the probes hybridize to their respective target sequences, if any, to form probe target sequence hybrids. Any hybrids formed between the probes and target sequences can then be detected as an indication of the presence of the any target sequences in the test sample.

In certain cases, such as when the target sequences are not in sufficient quantity for detection, primers and amplification reagents may be added to the test sample prior to or at the same time the probes are contacted with the test sample. Due to the nature of the probes and the target sequences with which they hybridize, often times a single set of amplification primers can be selected to amplify both target sequences. Specifically, primers can be selected such that they amplify the target sequence in such a fashion that the variation in the second target sequence will also be included in the amplification product if it is present. For example, primers capable of amplifying the factor V gene and the sequence containing the

factor V Leiden mutation include SEQ. ID. NO. 3 and SEQ. ID. NO. 4. Additionally, primers capable of amplifying the 3' untranslated region of the prothrombin gene and mutation to the 3' untranslated region of the prothrombin gene include SEQ. ID. NO. 14 and SEQ. ID. NO. 15.

Examples

Example 1
Sequences

A. Leiden Factor V Sequences : The following examples demonstrate detection of wild type and mutant Leiden Factor V using the DNA oligomer primers and probes herein provided. These DNA primers and probes are identified as SEQ. ID NO. 3, SEQ. ID NO. 4, SEQ. ID NO. 5, SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9, SEQ. ID NO. 10 and SEQ. ID NO. 11. A portion of a representative sequence of wild type Leiden Factor V is designated herein as SEQ. ID NO. 1, and a portion of the similar representative sequence of mutant Leiden Factor V is designated herein as SEQ. ID NO. 2. SEQ. ID NO. 2 differs from SEQ. ID NO. 1 at only one base, which is the point mutation that distinguishes mutant Leiden Factor V from wild type. SEQ. ID NO. 3 and SEQ. ID NO. 4 are specific for regions found in both wild type and mutant Leiden Factor V. SEQ. ID NO. 5 is specific for a region found in wild type Leiden Factor V only. SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9, SEQ. ID NO. 10 and SEQ. ID NO. 11 are specific for a region found in mutant Leiden Factor V only.

In the following examples, SEQ. ID NO. 3 and SEQ. ID NO. 4 are used as amplification primers for both wild type and mutant Leiden Factor V. SEQ. ID NO. 5 is used as an internal hybridization probe for the wild type Leiden Factor V amplification product. SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9, SEQ. ID NO. 10 and SEQ. ID NO. 11 are used as internal hybridization probes for the mutant Leiden Factor V amplification product.

B. Prothrombin Sequences: The following examples demonstrate detection of the wild type and mutant prothrombin genes using the DNA oligomer primers and probes herein provided. These DNA primers and probes are identified as SEQ. ID NO. 12, SEQ. ID NO. 13, SEQ. ID NO. 14, SEQ. ID NO. 15, SEQ. ID NO. 16, SEQ. ID NO. 17, SEQ. ID NO. 18, SEQ. ID NO. 19 and SEQ. ID NO. 20. A portion of a representative sequence of the wild type prothrombin

gene is designated herein as SEQ. ID NO. 12, and a portion of the similar representative sequence of the mutant prothrombin gene is designated herein as SEQ. ID NO. 13. SEQ. ID NO. 13 differs from SEQ. ID NO. 12 at only one base, which is the point mutation that distinguishes the mutant prothrombin gene from the wild type. SEQ. ID NO. 14 and SEQ. ID NO. 15 are specific for regions found in both the wild type and mutant prothrombin genes. SEQ. ID NO. 16 is specific for a region found in the mutant prothrombin gene only. SEQ. ID NO. 17, SEQ. ID NO. 18, SEQ. ID NO. 19 and SEQ. ID NO. 20 are specific for a region found in the wild type prothrombin gene only.

In the following examples, SEQ. ID NO. 14 and SEQ. ID NO. 15 are used as amplification primers for both the wild type and mutant prothrombin genes. SEQ. ID NO. 16 is used as an internal hybridization probe for the mutant prothrombin gene amplification product. SEQ. ID NO. 17, SEQ. ID NO. 18, SEQ. ID NO. 19 and SEQ. ID NO. 20 are used as internal hybridization probes for the wild type prothrombin gene amplification product.

Example 2
Preparation of Primers and Probes

A. wild type and mutant Leiden Factor V Primers Primers were designed to detect the target sequence of both wild type and mutant Leiden Factor V by oligonucleotide hybridization PCR. These primers were SEQ. ID NO. 3 and SEQ. ID NO. 4. Primer sequences were synthesized using standard oligonucleotide synthesis methodology. The SEQ. ID NO. 4 primer was haptenated with carbazole at the 5' end using standard cyanoethyl phosphoramidite coupling chemistry as described in U.S. Patent No. 5,424,414 incorporated herein by reference.

B. wild type and mutant Leiden Factor V Probes Probes were designed to hybridize with the amplified target sequence of wild type or mutant Leiden Factor V by oligonucleotide hybridization. These probes were SEQ. ID NO. 5 for wild type Leiden Factor V and SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9, SEQ. ID NO. 10 and SEQ. ID NO. 11 for mutant Leiden Factor V. Probe sequences were synthesized using standard oligonucleotide synthesis methodology. The SEQ. ID NO. 5 wild type probe was haptenated with adamantane at the 5'end, followed by 10 thymidines and blocked with phosphate at the 3' end. The SEQ. ID NO. 6, SEQ. ID NO. 7, SEQ. ID NO. 8, SEQ. ID NO. 9, SEQ. ID NO. 10 and SEQ. ID NO. 11 mutant probes were haptenated with dansyl at the 5 ' end, followed by 10 thymidines and blocked with phosphate at the 3' end. All syntheses used standard

cyanoethyl phosphoramidite coupling chemistry as described in U.S. Patent No. 5,464,746 (herein incorporated by reference).

C. wild type and mutant Prothrombin Primers Primers were designed to detect the target sequence of both the wild type and mutant prothrombin gene by oligonucleotide hybridization PCR. These primers were SEQ. ID NO. 14 and SEQ. ID NO. 15. Primer sequences were synthesized using standard oligonucleotide synthesis methodology. The SEQ. ID NO. 14 primer was haptenated with either carbazole or adamantane at the 5' end using standard cyanoethyl phosphoramidite coupling chemistry as described in U.S. Patent No. 5,424,414 incorporated herein by reference. The SEQ. ID NO. 15 primer was haptenated with adamantane at the 5'end by the same method.

D. wild type and mutant Prothrombin Probes Probes were designed to hybridize with the amplified target sequence of the wild type or mutant prothrombin gene by oligonucleotide hybridization. These probes were SEQ. ID NO. 16 for the mutant prothrombin gene and SEQ. ID NO. 17, SEQ. ID NO. 18, SEQ. ID NO. 19 and SEQ. ID NO. 20 for the wild type prothrombin gene. Probe sequences were synthesized using standard oligonucleotide synthesis methodology. The SEQ. ID NO. 16 mutant probe was either haptenated with 2 carbazoles at the 3' end, or haptenated with 2 dansyls at the 5'end, followed by 10 thymidines and blocked with phosphate at the 3' end. The SEQ. ID NO. 17, SEQ. ID NO. 18 and SEQ. ID NO. 19 wild type probes were haptenated with 2 adamantanes at the 5 ' end, followed by 10 thymidines and blocked with phosphate at the 3' end. Separately, both the SEQ. ID NO. 17 and SEQ. ID NO. 20 wild type probes were haptenated with 2 carbazoles at the 3' end. All syntheses used standard cyanoethyl phosphoramidite coupling chemistry as described in U.S. Patent No. 5,464,746 (herein incorporated by reference).

Example 3
Sample Preparation

Wild type, heterozygous and homozygous mutant Leiden Factor V and Prothrombin DNA was purified from 200 µl of each whole blood sample using the QIAamp® Blood Mini Kit (QIAgen, Inc., Valencia, CA) nucleic acid extraction procedure and column methodology as described by the manufacturer. The DNA samples were simultaneously wild type, heterozygous or homozygous mutant for both genes. The genotype of all samples was verified by sequencing. Purified DNA was quantitated by taking the absorbance reading at

260 nm using a spectrophotometer. The final concentration of the purified DNA was in the range of 50 to 25 ng/µl.

Example 4
Detection of Mutant vs. Wild Type Leiden Factor V using Offset Probes

The probes used to detect the single base pair mutation distinguishing mutant from wild type Leiden Factor V were a sense strand probe and an antisense strand probe, respectively, and so would be exact compliments of one another except at the single base pair mismatch. Thus they could also hybridize with one another rather than with their specific target amplified DNA. It was hypothesized that offsetting the probes slightly from one another might help minimize any hybridization between them. This was tested by deleting 2 bases from the 5' end of each probe.

Duplicates of each of the purified wild type, heterozygous and homozygous mutant Leiden Factor V/Prothrombin DNA samples were PCR amplified and detected using SEQ. ID NO. 3 and SEQ. ID NO. 4 primers with the SEQ. ID NO. 5,wild type probe and the SEQ. ID NO. 6 mutant probe. PCR was performed in buffer containing final concentrations of 50 mM Bicine (N,N,-bis[2-Hydroxyethyl]glycine), pH 8.1, 150 mM potassium, 8% w/v glycerol, 0.001% bovine serum albumin (BSA), 0.1 mM EDTA and 0.02% sodium azide. Recombinant *Thermus thermophilus* polymerase was used at a concentration of 5 units/reaction, with dNTPs (dATP, dGTP, dTTP and dCTP) present at a final concentration of 150 µM each. The SEQ. ID NO. 3 primer was used at a concentration of 120 nM, while the SEQ. ID NO. 4 primer (labeled with carbazole) was used at a concentration of 60 nM. The SEQ. ID NO. 5 wild type probe was present at a concentration of 40 nM, and the SEQ. ID NO. 6 mutant probe was present at a concentration of 30 nM. A final concentration of 3.25 mM Manganese chloride was used in a total reaction volume of 0.2 ml, with sample volume of 20 µl.

Reaction mixtures were amplified in an LCx® Thermal Cycler. Reaction mixtures were first incubated at 97°C for 4 minutes, followed by 45 cycles of PCR amplification at 94°C for 30 seconds then 55°C for 45 seconds. After the reaction mixtures were thermal cycled, the mixtures were maintained at 97°C for 5 minutes and probe oligo hybridization was accomplished by lowering the temperature to 15°C. Samples were held at 12°C for a minimum of 5 minutes, and thereafter until reaction products were analyzed and detected.

Reaction products were detected on the Abbott LCx® system (available from Abbott Laboratories, Abbott Park, IL). A suspension of anti-carbazole coated microparticles, an anti-adamantane antibody/alkaline phosphatase conjugate and an anti-dansyl antibody/β-

galactosidase conjugate (all available from Abbott Laboratories, Abbott Park, IL) were used in conjunction with the LCx® to capture and detect the reaction products. The enzyme substrates used were 4-methyl-umbelliferyl phosphate (MUP) and 7-β-D-galactopyranosyloxy coumarin-4-acetic acid-(2-hydroxyethyl) amide (AUG) with the rate of conversion of substrate to product measured and reported as counts/second/second (c/s/s).

The results in Table 1 show that while the wild type probe was able to detect both the homozygous wild type and the heterozygous DNA, and not the homozygous mutant DNA (all as expected), the mutant probe was unable to detect either the homozygous mutant or the heterozygous DNA.

TABLE 1

Purified DNA Genotype	Wild type probe LCx® rate (avg. c/s/s)	Mutant probe LCx® rate (avg. c/s/s)
Wild type	667	27
Heterozygous	475	32
Homozygous Mutant	78	39

Modifications were made to the mutant probe, replacing the 2 removed bases at the 5' end and adding additional bases to the 3'end either with (SEQ. ID NO. 7, A) or without (SEQ. ID NO. 11, E) a mismatch G for destabilization, or still deleting the 2 bases at the 5' end but adding 3, 4 or 5 bases to the 3' end (SEQ. ID NO. 8 (B), 9 (C) or 10 (D), respectively). The experimental procedure described above was repeated using each of these mutant probes instead of SEQ. ID NO. 6 in separate reactions with the SEQ. ID NO. 5 wild type probe. The results are shown in Table 2 below.

TABLE 2

Purified DNA Genotype	Wild Type / Mutant Probe Pairs: LCx® rate (avg. c/s/s)									
	WT	Mut A	WT	Mut B	WT	Mut C	WT	Mut D	WT	Mut E
WT	317	53	396	40	404	62	431	258	368	60
HT	240	219	244	298	244	378	259	530	284	210
HM	30	593	32	528	36	648	40	728	48	492

(WT = wild type; Mut = mutant; HT = heterozygous; HM = homozygous mutant)

All mutant probes performed better than the previous SEQ. ID NO. 6 mutant probe, with the ability to detect both the heterozygous and homozygous mutant DNA. With the exception of mutant probe D, the mutant probes also showed good specificity inasmuch as they did not detect wild type DNA. The wild type probe continued to perform well, detecting both wild type and heterozygous, but not homozygous mutant, DNA. The best signals were achieved using mutant probes C (SEQ. ID NO. 9) and D (SEQ. ID NO. 10). Thus, the best combination of probes for detecting either wild type or mutant Leiden Factor V DNA was using the wild type probe (SEQ. ID NO. 5), having a 2 base deletion at the 5' end, with mutant probe C (SEQ. ID NO. 9), also having a 2 base deletion at the 5' end but also containing 4 additional bases on the 3' end. This effectively offset the base pair mismatch between the wild type and mutant Leiden Factor V sequence, moving it from the middle to closer to the 5' end.

Thus, as a general matter, the offset probes were able to specifically hybridize to their appropriate amplified target sequences.

Example 5
Detection of Mutant vs. Wild Type Prothrombin

A. Using Sense Strand Probes Initial experiments to detect the wild type vs. mutant polymorphism on the prothrombin gene were performed using similar sense strand probes, one for the mutant gene (SEQ. ID NO. 16) and the other for the wild type gene (SEQ. ID NO. 20), in separate reactions. In addition to the one base pair difference at the wild type vs. mutant locus, the wild type probe also contained 2 additional base pairs at the 5' end.

Duplicates of the purified heterozygous and homozygous mutant Leiden Factor V/Prothrombin DNA samples were diluted 1:100, 1:1000 and 1:10,000 in Molecular Grade Water (5'-3', Inc., Boulder, CO), PCR amplified using SEQ. ID NO. 14 and SEQ. ID NO. 15 primers labeled with adamantane and detected using either the carbazole labeled SEQ. ID NO. 16 mutant probe or the carbazole labeled SEQ. ID NO. 20 wild type probe, in separate reactions. PCR was performed in buffer containing final concentrations of 50 mM Bicine (N,N,-bis[2-Hydroxyethyl]glycine), pH 8.1, 150 mM potassium, 8% w/v glycerol, 0.001% bovine serum albumin (BSA), 0.1 mM EDTA and 0.02% sodium azide. Recombinant *Thermus thermophilus* polymerase was used at a concentration of 5 units/reaction, with dNTPs (dATP, dGTP, dTTP and dCTP) present at a final concentration of 150 µM each. The primers were used at a concentration of 250 nM each, with the probes present at a concentration of 5 nM each. A final concentration of 3.25 mM Manganese chloride was used in a total reaction volume of 0.2 ml, with sample volume of 20 µl. Duplicates of Poly-A at 20 ng/µl were run as a negative control.

Reaction mixtures were amplified in an LCx® Thermal Cycler. Reaction mixtures were first incubated at 95°C for 1 minute, then 56°C for 30 seconds, and 97°C for 2 minutes, followed by 40 cycles of PCR amplification at 95°C for 40 seconds then 58°C for 60 seconds. After the reaction mixtures were thermal cycled, the mixtures were maintained at 72°C for 5 minutes, then 97°C for 5 minutes, with probe oligo hybridization accomplished by lowering the temperature to 15°C for 2 minutes. Samples were held at 4°C thereafter until reaction products were analyzed and detected.

Reaction products were detected on the Abbott LCx® system (available from Abbott Laboratories, Abbott Park, IL). A suspension of anti-carbazole coated microparticles and an anti-adamantane antibody/alkaline phosphatase conjugate (all available from Abbott Laboratories, Abbott Park, IL) were used in conjunction with the LCx® to capture and detect the reaction products. The enzyme substrate used was 4-methyl-umbelliferyl phosphate (MUP) with the rate of conversion of substrate to product measured and reported as counts/second/second (c/s/s).

As can be seen in Table 3 below, while the mutant probe detected both the heterozygous and the homozygous mutant samples, the wild type probe also detected both samples, though it should only react with the heterozygous sample and not the homozygous mutant. Thus, this probe combination, with both probes from the sense strand, was not be able to distinguish the wild type from the mutant polymorphism of the prothrombin gene.

TABLE 3

Purified DNA Genotype (Sample Dilution)	Wild type sense probe LCx® rate (avg. c/s/s)	Mutant sense probe LCx® rate (avg. c/s/s)
Heterozygous 1:100	850	553
1:1000	594	357
1:10,000	195	148
Homozygous Mutant 1:100	731	642
1:1000	485	441
1:10,000	172	111
Poly A – Negative Control	24	17

B. Using Sense vs. Antisense Probes Since probes from the sense strand failed to differentiate the wild type from the mutant allele of the prothrombin gene, the wild type

probe was moved to the antisense strand to determine if this would facilitate discrimination of the 2 alleles.

This experiment was performed as described in Example 5.A. above, except using the carbazole labeled SEQ. ID NO. 17 wild type probe instead of the SEQ. ID NO. 20 probe, and testing duplicates of samples at the 1:100 dilution only. Thermal cycling conditions were also modified as follows: reaction mixtures were first incubated at 97°C for 2 minutes, followed by 40 cycles of PCR amplification at 95°C for 40 seconds then 58°C for 60 seconds. After the reaction mixtures were thermal cycled, the mixtures were maintained at 97°C for 5 minutes, with probe oligo hybridization accomplished by lowering the temperature to 15°C for 2 minutes. Samples were held at 15°C thereafter until reaction products were analyzed and detected.

TABLE 4

Purified DNA Genotype (Sample Dilution)	Wild type sense probe LCx® rate (avg. c/s/s)	Mutant sense probe LCx® rate (avg. c/s/s)
Heterozygous 1:100	586	792
Homozygous Mutant 1:100	172	938
Poly A – Negative Control	17	19

The results in Table 4 above, show that moving the wild type probe to the antisense strand allows discrimination between the wild type and mutant alleles to occur, with the wild type probe now showing a much higher detection signal with the heterozygous sample, containing one wild type allele, than with the homozygous mutant sample. The mutant probe also maintains its ability to detect both heterozygous and homozygous mutant samples. Additionally, the mutant probe, under these conditions, gives a slightly higher signal with the homozygous mutant sample, containing two mutant alleles, than with the heterozygous sample, with only one mutant allele, vs. what was seen in the experiment in Example 5.A.

C. Using Offset Probes Although discrimination of wild type and mutant prothrombin was achieved using one probe on the sense strand and the other on the antisense strand, in Example 5.B. above, the ability to combine both probes in a single reaction mixture, so that a sample would only have to be tested once to determine the genotype, would be advantageous. This experiment also used the sense (mutant)/antisense (wild type) probes in separate reactions, requiring two tests to be run for each sample to determine the sample's genotype. However, since the sense/antisense probes would be exact compliments of one another,

except at the single base pair mismatch, they could also hybridize with one another rather than with their specific target amplified DNA. Thermal cycling conditions were modified to help prevent this, and, additionally, the strategy that had been employed with the Leiden Factor V probes, offsetting the probes from one another around the base pair mismatch, was utilized.

The probe to detect the mutant allele of the prothrombin gene (SEQ. ID NO. 16) was the same as that used in Examples 5.A. and B. above, and contained the base pair mismatch in the center (base 6 of 13). Three wild type probes were tested with this mutant probe: wild type probe F (SEQ. ID NO. 19) was complementary to the mutant probe except for the single base pair mismatch; wild type probe G (SEQ. ID NO. 17) contained 2 additional bases at the 3' end; and wild type probe H (SEQ. ID NO. 18) contained 2 additional bases at the 3' end and deleted 2 bases from the 5' end, creating a 2 base overhang on each probe's 3' end.

Duplicates of each of the purified wild type, heterozygous and homozygous mutant Leiden Factor V/Prothrombin DNA samples were PCR amplified and detected as in Example 4 except using SEQ. ID NO. 14 and SEQ. ID NO. 15 primers, and the SEQ. ID NO. 16 mutant probe with each of the 3 wild type probes described above in separate reactions (i.e. the mutant probe and one of each of the wild type probes were used together in the same reaction mixture). The reaction conditions used were those described in Example 4 above, except for the primer and probe concentrations which were as follows: the SEQ. ID NO. 14 primer (labeled with carbazole) was used at a concentration of 200 nM, the SEQ. ID NO. 15 primer was used at a concentration of 150 nM; the SEQ. ID NO. 16 mutant probe was used at a concentration of 70 nM, and either the SEQ. ID NO. 17 (G), 18 (H) or 19 (F) wild type probe was present in each reaction mixture at a concentration of 45 nM. The results from this experiment are shown in Table 5 below.

TABLE 5

Purified DNA Genotype	Wild Type / Mutant Probe Pairs: LCx® rate (avg. c/s/s)					
	WT F	Mut	WT G	Mut	WT H	Mut
Wild type	277	67	302	59	365	54
Heterozygous	215	400	206	403	204	290
Homozygous Mutant	59	720	88	703	59	444

(WT = wild type; Mut = mutant)

All wild type/mutant probe pairs showed specific detection of the appropriate wild type or mutant allele with the wild type probes detecting both homozygous wild type and heterozygous prothrombin samples but not detecting homozygous mutant prothrombin DNA

as positive. The mutant probe detected both homozygous mutant and heterozygous prothrombin samples but did not detect homozygous wild type prothrombin samples as positive. As expected, all probes detected the heterozygous samples since they contain one wild type and one mutant allele, and these probes did so in a dose dependent manner. Thus, all probes showed excellent specificity.

The wild type probe (F) that was completely complementary to the mutant probe (except at the base pair mismatch) worked, but gave the lowest signal with the homozygous wild type sample. The wild type probe that showed the best signal when detecting the wild type allele was mutant probe H (SEQ. ID NO. 18), the probe combination giving a 2 base overhang at both ends. This probe combination also showed the best balance (equivalency) between mutant and wild type positive signals.

While the invention has been described in detail and with reference to specific embodiments, it will be apparent to one skilled in the art that various changes and modifications may be made to such embodiments without departing from the spirit and scope of the invention.

Claims

(1) A method for detecting at least one of two related target nucleic acid sequences in a test sample comprising:

a) contacting the test sample with a first and second probe wherein,
   (i) the probes are completely complementary to the each other except for at least one mismatch,
   (ii) the first probe is more complementary to a sense strand of a first target sequence than to the second probe,
   (iii) the second probe is more complementary to an anti-sense strand of a second target sequence than to the first probe, and
   (iv) the probes hybridize to each other at an ambient temperature;

b) hybridizing at least the first or second probe to the first or second target sequence to form at least one target sequence/probe hybrid; and

c) detecting the hybrid as an indication of the presence of at least one target sequence in the test sample.

(2) The method of claim 1 wherein the second target sequence is a variant form of the first target sequence.

(3) The method of claim 2 wherein the variant form of the first target sequence includes a single nucleotide polymorphism.

(4) The method of claim 3 wherein the probes are SEQ. ID. NO. 5 and a member of the group consisting of SEQ. ID. NO. 7, SEQ. ID. NO. 8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, and SEQ. ID. NO. 11.

(5) The method of claim 3 wherein the probes are SEQ. ID. NO.16 and a member of the group consisting of SEQ. ID. NO. 17, SEQ. ID. NO. 18, SEQ. ID. NO. 19, and SEQ. ID. NO. 20.

(6) The method of claim 1 further comprising the steps of: providing the test sample with a primer set, and amplification reagents; and amplifying any target sequences in

the test sample; prior to or at the same time as the test sample is contacted with the first and second probes.

(7) The method of claim 6 wherein the primer set is selected from the group consisting of SEQ. ID. NO. 3 and SEQ. ID. NO. 4; and SEQ. ID. NO. 14 and SEQ. ID. NO. 15.

(8) The method of claim 1 wherein the first and second probes hybridize to each other such that the first probe has a 3' overhang.

(9) The method of claim 8 wherein the second probe has a 3' overhang.

(10) The method of claim 8 wherein the overhang is between 1 and 5 nucleotides long.

(11) A composition of matter comprising: a first and second probe wherein,

(a) the probes are completely complementary to the each other except for at least one mismatch,

(b) the first probe is more complementary to a sense strand of a target sequence than to the second probe,

(c) the second probe is more complementary to an anti-sense strand of a second target sequence than to the first probe, and

(d) the probes hybridize to each other at an ambient temperature.

(12) The composition of claim 11 wherein the probes individually are between 10 and 100 nucleotides long.

(13) The composition of claim 12 wherein the first and second probes hybridize to each other such that the first probe has a 3' overhang.

(14) The method of claim 13 wherein the second probe has a 3' overhang.

(15) The method of claim 13 wherein the overhang is between 1 and 5 nucleotides long.

(16) The composition of claim 11 wherein the mismatch is a single nucleotide polymorphism.

(17) The method of claim 3 wherein the probes SEQ. ID. NO. 5 and a member of the group consisting of SEQ. ID. NO. 7, SEQ. ID. NO. 8, SEQ. ID. NO. 9, SEQ. ID. NO. 10, and SEQ. ID. NO. 11.

(18) The method of claim 3 wherein the probes are SEQ. ID. NO.16 and a member of the group consisting of SEQ. ID. NO. 17, SEQ. ID. NO. 18, SEQ. ID. NO. 19, and SEQ. ID. NO. 20.

SEQUENCE LISTING

<110> Abbott Laboratories
Solomon, Natalie A.
Erickson, Dwight D.
Ziegler, Sharon R.

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